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## Novel biosynthetic approaches to the production of unnatural amino acids using transaminases

Paul P. Taylor, David P. Pantaleone, Richard F. Senkpeil and Ian G. Fotheringham

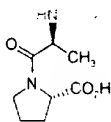
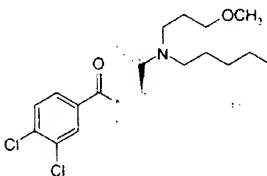
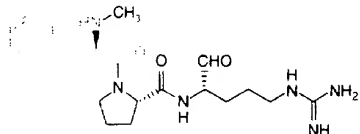
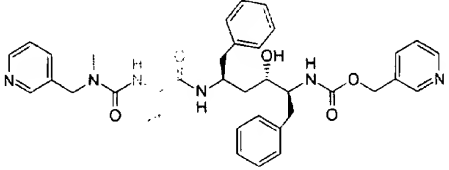
Transaminase enzymes are being increasingly applied to the large-scale synthesis of unnatural and nonproteinogenic amino acids. Typically displaying relaxed substrate specificity, rapid reaction rates and lacking the need for cofactor regeneration, they possess many characteristics that make them desirable as effective biocatalysts. By judiciously combining the transaminase reaction with additional enzymatic steps, this approach can be used very efficiently to prepare a broad range of D- and L-amino acids.

**T**ransaminases have been studied extensively since their discovery over 60 years ago and have frequently been investigated in biotransformation approaches for the production of natural amino acids and chiral amines<sup>1–3</sup>. Recently, they have been widely applied in the large-scale biosynthesis of unnatural amino acids<sup>4,5</sup>, which are in increasing demand by the

pharmaceutical industry for peptidomimetic and other single-enantiomer drugs (Table 1). The existence of transaminases with broad substrate specificity for the synthesis of either D- or L-amino acids makes these enzymes attractive for this type of application, in which the desired products are structurally diverse. The process is often most effective as a biotransformation when the transaminase reaction is coupled to additional enzymatic steps through the use of multiple cloned genes combined in a single organism. Alternatively, it is possible to modify naturally occurring biochemical pathways to bring about the biosynthesis of unnatural

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Table 1. Examples of unnatural amino acids in peptidomimetic drugs in development

Therapeutic area	Target	Unnatural amino acid	Structure <sup>a</sup>	Ref.
Hypertension	Angiotensin-converting enzyme (ACE)	L-Homophenylalanine		6
Bowel disorder	CCK-receptor antagonist	D-Glutamate		7
Thrombosis	Thrombin	D-Phenylalanine		8
AIDS	HIV protease	L-tert-Leucine		9

<sup>a</sup>Unnatural amino acids shown in grey.

or nonproteinogenic (other than the 20 naturally occurring) amino acids through direct microbial fermentation. Examples of both types of process are given below.

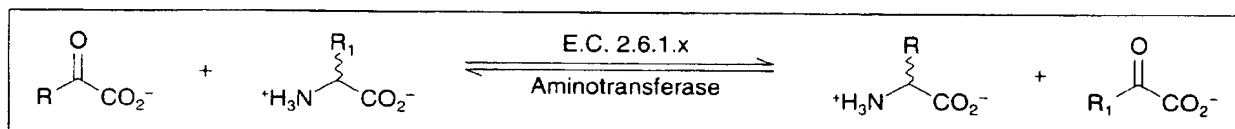
### General properties of aminotransferases

L- $\alpha$ -Amino acid transaminases (LATs) are ubiquitous in nature, being involved (directly or indirectly) in the biosynthesis of most natural amino acids. D-Amino acid transaminases (DATs) have been identified in a number of bacterial species, most notably the *Bacilli*, in which they provide D-amino acids for peptidoglycan and secondary metabolite biosynthesis<sup>10-14</sup>. The reaction mechanism is well understood, with a pyridoxal 5'-phosphate cofactor shuttling between pyridoxal and pyridoxamine forms in the reversible transfer of amino and keto groups between an amino-acid- $\alpha$ -keto-acid substrate pair to generate a new amino acid and keto acid (Fig. 1).

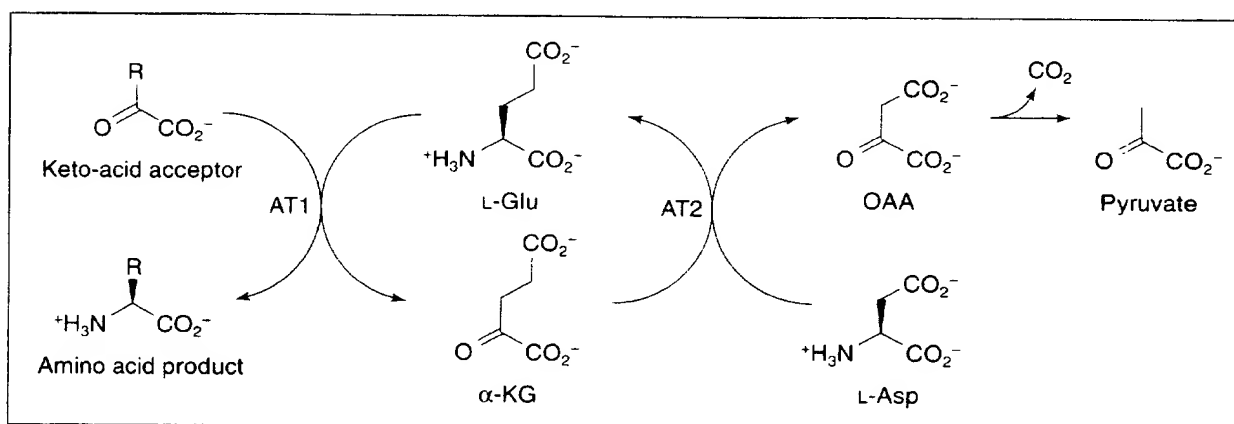
### The problem of reaction equilibrium

Although both types of transaminases have appropriate characteristics to function as commercial biocatalysts, with rapid reaction rates, broad substrate specificity and no requirement for external cofactor regeneration, the frequently cited drawback is the equilibrium constant for the reaction. This is typically close

to one, which can often compromise amino acid product yield, isolation and recovery. Earlier applications of LATs have addressed this issue by coupling two aminotransferases (Fig. 2). The first aminotransferase utilizes L-glutamate as the amino donor, while the second aminotransferase uses L-aspartate. L-Aspartate yields oxaloacetate upon transamination, which spontaneously decarboxylates to form pyruvic acid and carbon dioxide to favour the formation of the amino acid product. Elegant reaction schemes have previously been devised to optimize this process<sup>15,16</sup>. The introduction of additional enzymes, such as acetolactate synthase (ALS), similarly offers the potential to drive the reaction to completion (Fig. 3). ALS catalyses the dimerization of pyruvate to form acetolactate, which spontaneously decarboxylates to acetoin, thereby preventing transamination to alanine; this simplifies the recovery of the amino acid product<sup>5</sup> and also shifts the equilibrium of the transaminase reaction during the production of unnatural amino acids. The use of ALS is equally applicable to the synthesis of the D- or L-isomer by using different transaminase-encoding genes<sup>5</sup>. These coupled reaction schemes can be achieved with a single immobilized recombinant microbial strain containing the cloned genes for each of the enzymes. Other, more complex, coupled reactions for the production of D-amino acids will be described in more detail below.

**Figure 1**

General aminotransferase reaction showing the interconversion of keto and amino acids; the stereochemistry desired is based on the choice of enzyme and the amino donor. When the amino donor has the L configuration and an L-amino acid transaminase (LAT) is used, the product amino acid will have an L configuration, and when the amino donor has the D configuration and a D-amino transferase (DAT) is used, the product amino acid will have a D configuration.

**Figure 2**

A keto-acid acceptor is converted into the L-amino acid product using aminotransferase 1 (AT1) with L-glutamic acid (L-Glu) as the amino donor. The corresponding  $\alpha$ -ketoglutarate ( $\alpha$ -KG) is then converted back to L-Glu using L-aspartic acid (L-Asp) as the amino donor by aminotransferase 2 (AT2), accompanied by the formation of oxaloacetic acid (OAA). Spontaneous decarboxylation of OAA occurs to form pyruvate, thus driving the reaction beyond equilibrium.

### Supply of $\alpha$ -keto acids

Many  $\alpha$ -keto-acid substrates are available by chemical synthesis, but these are often the most expensive component in the reaction. Fortunately, the high value of the final product often offsets the relatively high substrate costs. In the case of the DAT reaction, keto acids are also accessible by enzymatic methods (such as L-amino acid deaminases), which are able to generate keto-acid substrates from inexpensive L-amino acids. For example, the L-amino acid deaminase (L-AAD) from *Proteus myxofaciens* (which has been cloned and sequenced) can deaminate a wide range of amino acids to the corresponding keto acids. Most importantly, it does not deaminate L-aspartate, L-glutamate or L-alanine, which, when racemized, can serve as amino donors in the DAT reaction (P. P. Taylor and D. P. Pantaleone, unpublished).

### Production of unnatural amino acids using L-aminotransferases

The most commonly employed LATs used for L-amino acid synthesis are the transaminases of *Escherichia coli*, most of which have been cloned and overexpressed. They are generally used in whole-cell or immobilized systems for industrial processes and include: aspartate aminotransferase (E.C. 2.6.1.1), branched-chain aminotransferase (E.C. 2.6.1.42) and tyrosine aminotransferase (E.C. 2.6.1.5). Below are details of each specific enzyme, primarily from *E. coli*, along with a description of the unnatural amino acids the enzyme has been used to synthesize (Fig. 4a and Table 2).

One of the most widely studied enzymes in the transaminase class is aspartate aminotransferase<sup>1</sup>. The *E. coli* enzyme, encoded by the *aspC* gene, is an 88-kDa protein that is active as a dimer. Although it has been described in the preparation of a number of natural amino acids, its use in the synthesis of unnatural amino acids is relatively recent<sup>17</sup>. Another industrially important unnatural amino acid, L-homophenylalanine, was synthesized with a 95% conversion yield using an aspartate aminotransferase from *Paracoccus denitrificans*<sup>18</sup>.

The branched-chain aminotransferase of *E. coli* is a hexamer composed of identical 33.9-kDa subunits<sup>19</sup>. Its three-dimensional X-ray crystallographic structure has recently been published, giving insights into the determinants of the keto-acid substrate specificity for this enzyme<sup>20</sup>. Prior to this, however, it had already been used to prepare L-*tert*-leucine from the keto acid trimethylpyruvate<sup>21</sup> and also many other unnatural amino acids<sup>16</sup>.

Tyrosine aminotransferase, encoded by the *tyrB* gene, catalyzes the final step in tyrosine biosynthesis. It transaminates *p*-hydroxyphenylpyruvate to tyrosine, using L-glutamate as the amino donor. This enzyme has also been used to prepare the unnatural herbicide amino acid L-phosphinothricin<sup>21</sup>, several different L-thienylalanines<sup>22,23</sup> and L-2-aminobutyric acid<sup>5</sup>.

In addition to the unnatural amino acids listed above, other non-proteinogenic amino acids can be synthesized using this transaminase technology. These include, straight-chain alkyl (norleucine, norvaline), diacid (2-aminoadipate), branched-chain

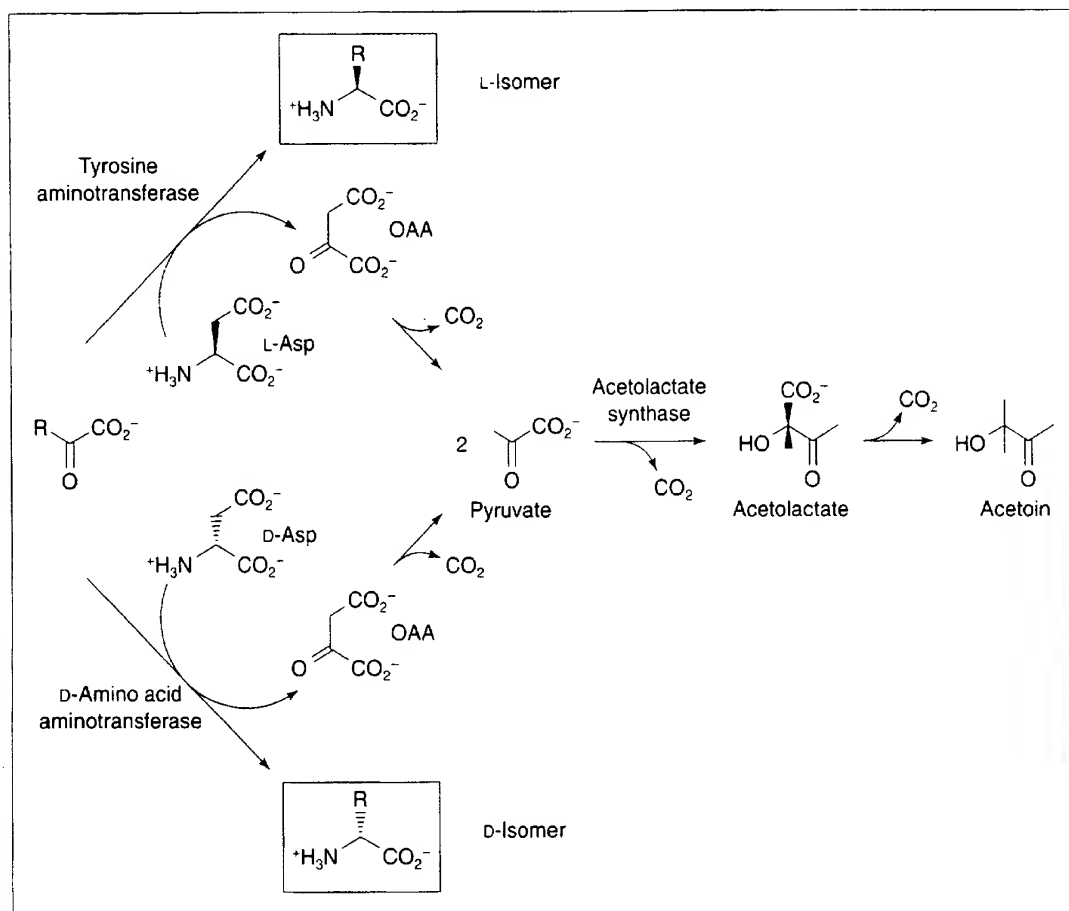


Figure 3

Preparation of either the L or the D isomer of amino acids using the respective amino donors and transaminases coupled to acetolactate synthase (ALS) to shift the reaction equilibrium. The oxaloacetate (OAA) that is formed during the transaminase reaction spontaneously decomposes to pyruvate, which is then converted to acetolactate by ALS, followed by spontaneous decarboxylation to acetoin. This effectively drives the reaction towards product formation and prevents pyruvate from being transaminated to L-alanine.

( $\gamma$ -methyl leucine), aromatic ( $\alpha$ -amino-2-furanacetic acid, phenylglycine, naphthylalanine) and bifunctional (2-amino-4-oxopentanoic acid) amino acids (R. F. Senkpeil *et al.*, unpublished). The wide variety of amino acids shown here serves to illustrate the broad industrial applicability of this technology.

#### Production of unnatural amino acids using bacterial D-aminotransferases

Unlike the LATs, very few DATs (E.C. 2.6.1.21) have been extensively studied. One of the best understood is that from *Bacillus* sp. YMI, whose gene has been cloned and sequenced<sup>24</sup> and whose crystal structure has been solved<sup>25</sup>. Recently, the *dat* genes from *Staphylococcus haemolyticus*<sup>26</sup>, *Bacillus licheniformis*<sup>27</sup> and *Bacillus sphaericus* (I. G. Fotheringham *et al.*, unpublished) have been cloned, sequenced and overexpressed in *E. coli*. These factors, plus their broad substrate specificity, make them ideal candidates for use in the production of D-amino acids (Table 2).

As with the LATs, the DATs have an equilibrium constant near unity. Fortunately, many of the procedures devised to optimize product formation with the LATs are also applicable to the DATs. Product insolubility, high substrate concentrations, unstable

keto-acid products (e.g. oxaloacetate) and the removal of the keto-acid product enzymatically (e.g. conversion of pyruvate to acetolactate) can be used to alter the equilibrium of the DAT reaction in favour of D-amino acid product. The supply of the D-amino acid donor can also be problematical because most D-amino acids are either unavailable as commodity chemicals or expensive. This problem is generally solved by using an L-amino acid such as L-aspartate, L-glutamate or L-alanine, which is converted *in situ* to a racemic mix using an appropriate racemase. The DNA sequences of these racemases are available in the GenBank DNA database (NCBI, Bethesda, MD, USA) and the genes are easily obtainable using PCR.

One of the simplest and most cost-effective routes to D-amino acids is shown in Fig. 3. Here, a whole-cell bioconversion reaction is driven to completion by the removal of pyruvate using ALS. This has been successfully applied to the production of a number of D-amino acids, including D-2-aminobutyrate<sup>5</sup> and D-glutamate (Fig. 4b)<sup>28</sup>. Theoretically, this route is applicable to the production of other D-amino acids, as long as the keto acid is not a substrate for ALS and either D-aspartate or D-alanine can be used as the amino donor.

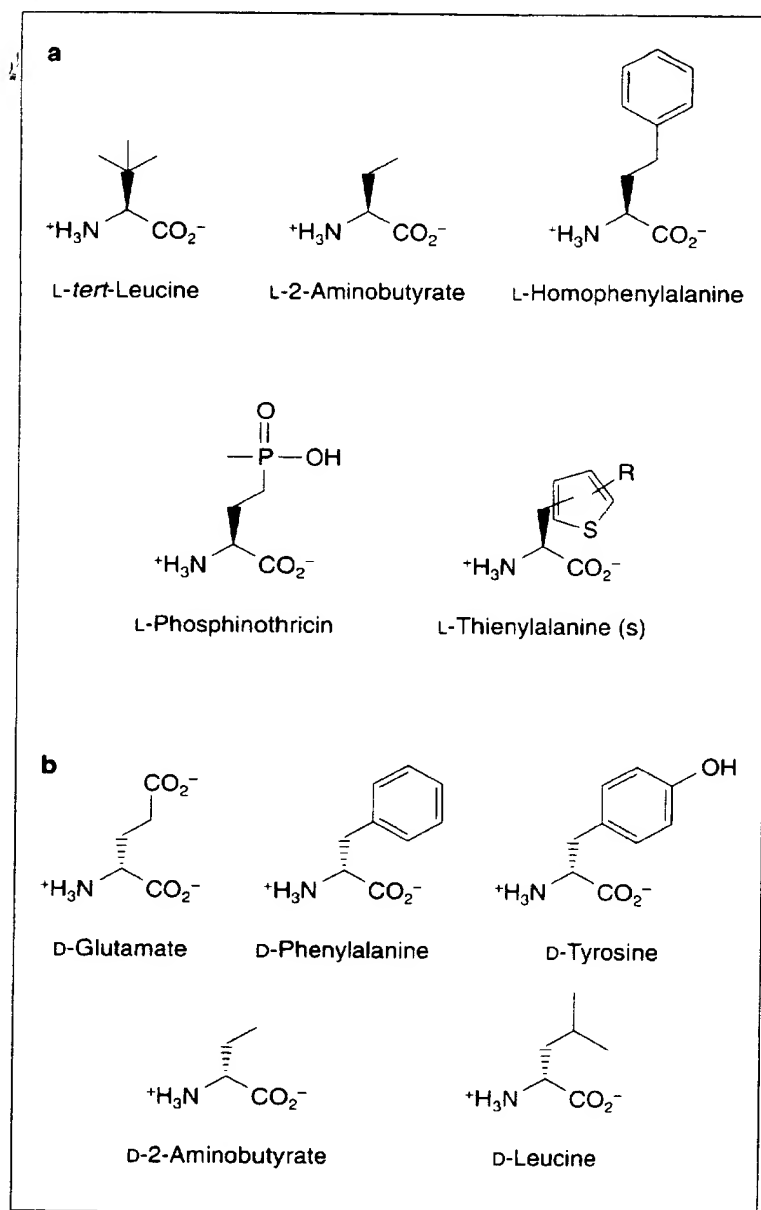


Figure 4

(a) Structures of unnatural L-amino acids prepared via transamination using L-amino acid transaminases. (b) Structures of D-amino acids prepared similarly using D-amino acid transaminases.

An elegant reaction scheme for the efficient production of a number of D-amino acids has been developed by Soda and colleagues<sup>29-31</sup>. Their optimized procedure uses a thermostable DAT from a *Bacillus* sp. in a coupled reaction with L-alanine dehydrogenase<sup>32</sup> and alanine racemase<sup>33</sup>, such that pyruvate is recycled to D-alanine via L-alanine (Fig. 5). It also requires an NADH-regeneration system, which is supplied by the action of a cloned, thermostable formate dehydrogenase<sup>34</sup>. As the dehydrogenation step is irreversible, the whole reaction proceeds towards 100% completion. All of the enzymes used were cloned and expressed in a single *E. coli* strain, thereby simplifying the reaction. The overall procedure works extremely well for D-glutamate and D-leucine (Fig. 4b), although other keto acids tested showed poor yields and/or poor enantiomeric excess. These problems were attributed to a number of causes, including reaction of the L-alanine dehydrogenase with a number of the keto acids tested, racemization of some of the amino acid products by the thermostable alanine racemase and transamination of keto acids by the host's L-transaminases. Other drawbacks include the need for relatively low substrate concentrations (by industrial standards) and the high cost of the keto-acid supply.

A flexible, modular approach to the production of D-amino acids is currently being developed that addresses both D-amino-acid-donor and the  $\alpha$ -keto-acid-acceptor supply but does not require exogenous regeneration of cofactors (P. P. Taylor *et al.*, unpublished); a generic depiction of this approach is shown in Fig. 6. The key step is the addition of L-AAD from *P. myxofaciens*, which is used to produce the  $\alpha$ -keto acid *in situ* by either feeding the L-amino acid substrate or using a strain that has been metabolically engineered to overproduce the requisite L-amino acid. In either case, the deamination takes place intracellularly. The D-amino donor is supplied as the L isomer, followed by racemization with an appropriate intracellular racemase. The two substrates are then converted by a cloned DAT to the corresponding D-amino acid. Because the reaction is carried out under fermentative conditions, the equilibrium is shifted towards 100% completion by the catabolism of the  $\alpha$ -keto product and regeneration of the amino donor by the cellular LATs. This system can be used to produce D-amino acids with high enantiomeric excess even in the presence of the host organism's endogenous L-transaminases. An example of this

Table 2. Aminotransferases used in unnatural amino acid processes

Transaminase	Abbreviation	Gene	Source	Compound(s)
Aspartate	AAT	<i>aspC</i>	<i>Escherichia coli</i>	L-Homophenylalanine
Branched-chain	BCAT	<i>ilvE</i>	<i>Escherichia coli</i>	L- <i>tert</i> -Leucine
Tyrosine	TAT	<i>tyrB</i>	<i>Escherichia coli</i>	L-2-Aminobutyrate
				L-Phosphinothricin
				L-Thienylalanine(s)
D-Amino acid	DAT	<i>dat</i>	<i>Bacillus</i> sp. YM1	D-Glutamate
			<i>Bacillus sphaericus</i>	D-Leucine
				D-Phenylalanine
				D-Tyrosine
				D-2-Aminobutyrate

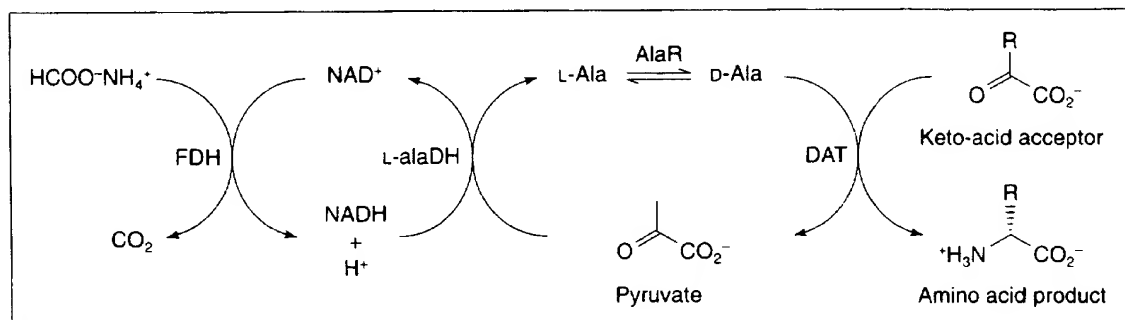


Figure 5

Preparation of D-amino acids using a coupled D-aminotransferase (DAT) and L-alanine dehydrogenase (L-aladH) with the formate dehydrogenase (FDH) cofactor-regeneration system. Alanine racemase (AlaR) is also used to racemize alanine to provide the amino donor.

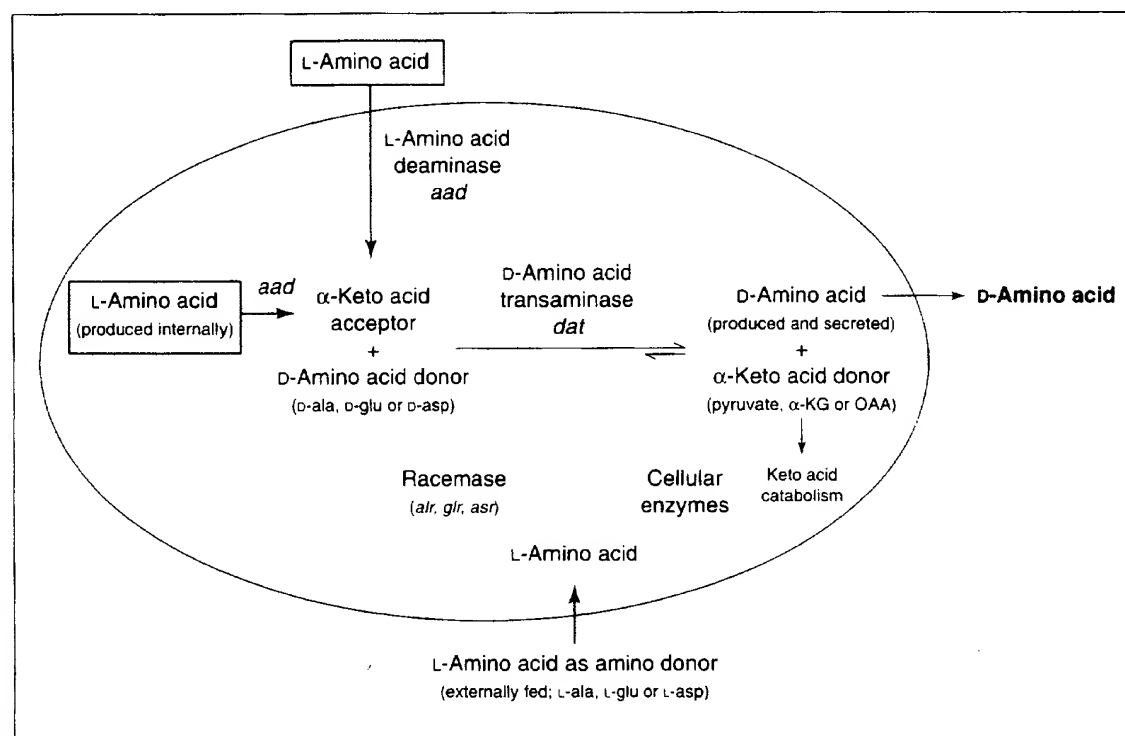


Figure 6

Diagrammatic representation of a bacterial cell producing D-amino acids using a fermentation or fed-fermentation approach. L-Amino acid deaminase (L-AAD) is used to produce the  $\alpha$ -keto-acid acceptor from the appropriate L-amino acid, which can be either fed externally (dark grey box) or produced internally (light grey box) by metabolic deregulation; the amino donor can, in a similar fashion, be supplied either externally (dark grey arrow) or internally (light grey arrow). An appropriate racemase is used to convert L-alanine, L-glutamate or L-aspartate to their respective D-amino acid donors. The overall reaction is shifted towards product by the removal of the  $\alpha$ -keto product of the reaction by cellular catabolism. Abbreviations: *aad*, L-amino acid deaminase gene;  $\alpha$ -KG,  $\alpha$ -ketoglutarate; *alr*, alanine racemase gene; *asr*, aspartate racemase gene; *dat*, D-amino acid transaminase gene; *glr*, glutamate racemase gene; OAA, oxaloacetic acid.

process is the production of D-phenylalanine (Fig. 4b), which can be produced in high yields with 99% enantiomeric excess using a combination of the approaches described above<sup>35,36</sup>. D-Tyrosine can also be synthesized in much the same way, using a fed-fermentation process (feeding L-tyrosine and L-alanine), but with improved overall yields owing to the insolubility of the final product (R. F. Senkpeil, unpublished). The promiscuous substrate range of L-AAD and DAT means that this type of approach can be used to convert a wide range of L-amino acids to their D-amino acid counterparts.

#### Other aminotransferases

The enzyme 4-aminobutyrate-2-ketoglutarate transaminase (E.C. 2.6.1.19) has also been used to prepare the herbicide L-phosphinothricin<sup>47,38</sup>. Most recently, this enzyme has been coupled with aspartate aminotransferase to drive the equilibrium in a similar way to the reaction shown in Fig. 2<sup>39</sup>.

A novel D-phenylglycine aminotransferase from *Pseudomonas stutzeri* ST-210 has recently been purified and characterized<sup>40</sup>. This transaminase is unusual in that it has a very narrow substrate range and causes a

stereoinversion of the transaminated amino acid. Specifically, it catalyses the reversible transamination of D-phenylglycine or D-4-hydroxyphenylglycine, using  $\alpha$ -ketoglutarate as the amino acceptor, to form L-glutamate. Although the synthesis of D-phenylglycine was clearly demonstrated using this enzyme, the low cost of the final product and the relatively high cost of the keto-acid precursors preclude its use in industrial production.

### Conclusions and future perspectives

The processes above illustrate a number of biotransformations carried out by bacterial transaminases acting in concert with additional biocatalysts in recombinant strains. The breadth of application is due in part to the inherent versatility of transaminases, but also to the detailed characterization of transaminase-encoding genes from a variety of microorganisms. Homology studies have indicated a high degree of primary-sequence similarity between many of the LATs<sup>41</sup>, and the recent cloning of genes encoding DATs has indicated similar sequence conservation within that family<sup>26,27</sup>. High-resolution crystal structures have been published for both enzyme classes, allowing site-directed-mutagenesis studies to probe their catalytic mechanism and the determinants of substrate specificity<sup>25,42</sup>. With the continuing advancement of gene-isolation and -mutagenesis procedures such as error-prone PCR and gene shuffling, it is likely that novel transaminases and variants of the known enzymes will further increase their application in the biosynthesis of unnatural amino acids.

Demand for chiral chemicals by the pharmaceutical industry is still in its infancy. It has been driven mainly by the advent of a combinatorial-chemistry approach to drug discovery and the rational design of a number of peptide-based pharmaceuticals. The approaches discussed above have outlined some of the ways in which transaminase technology has addressed this growing need for unnatural amino acids. Although it is clear that no one specific method for unnatural amino acid synthesis is applicable to all products, it is now possible to integrate many of the elements of current processes to form efficient and cost effective routes to novel chiral compounds.

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